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# INHIBITION OF ALKALINE PHOSPHATASE BY SEVERAL DIURETICS

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## Summary

Acetazolamide, furosemide, ethacrynic acid and chlorothiazide, diuretics of considerable structural diversity, inhibit alkaline phosphatase. The inhibition is reversible and the mechanism is of the mixed type, having both competitive and non-competitive characteristics. $K_i$ is calculated to be 8.4, 7.0, 2.8 and 0.1 mmol/l for acetazolamide, furosemide, ethacrynic acid and chlorothiazide, respectively. Chlorothiazide is a much more potent inhibitor of alkaline phosphatase than the other three diuretics. The combination of ethacrynic acid and cysteine, itself an alkaline phosphatase inhibitor, is less inhibitory than ethacrynic acid alone. Rat and human kidney alkaline phosphatase are equally sensitive to chlorothiazide, ethacrynic acid and furosemide.



## Introduction

We have recently described [1] the inhibition of human alkaline phosphatase (AP) by a group of sulfonamides which includes the diuretics acetazolamide (Diamox®) and furosemide (Lasix®). The methylated xanthine diuretic, theophylline, has been shown to inhibit AP in vitro [2–4]. Although dissimilar in structure, furosemide and ethacrynic acid are considered to be much alike in their pharmacologic properties [5]. Ethacrynic acid depresses calcium ion accumulation in rat kidney cortex mitochondria, and it is thought that this process is involved in the physiologic regulation of calcium and active transcellular transport in renal tubules [6]. Chlorothiazide (Diuril®) also affects calcium metabolism, causing hypercalcemia as well as inhibiting phosphodiesterase [7]. We studied the effects of furosemide, ethacrynic acid, acetazolamide and chlorothiazide on the kinetics of AP activity to elucidate the mechanisms of

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inhibition. Because of the reported [8,9] difference in diuretic effect of ethacrynic acid in rats and humans, we compared the effects of ethacrynic acid, furosemide and chlorothiazide on activity from rat and human kidney.

A synergistic interaction between cysteine and ethacrynic acid in the inhibition of Na-K-ATPase in the thick ascending limb of Henle's loop has been reported [10]. Burg attributed the amelioration of other toxic effects of ethacrynic acid to the formation of a less toxic or non-toxic cysteine-ethacrynic acid adduct with enhanced ability to block chloride transport. Because cysteine is a potent AP inhibitor, we tested the interaction of both agents to determine whether the effects on AP are synergistic, additive, or antagonistic.

## Materials and methods

Chlorothiazide was manufactured by the research laboratories of Merck, Sharp and Dohme, West Point, PA, U.S.A.; furosemide was procured from Hoechst-Roussel, Somerville, NJ, U.S.A.; ethacrynic acid was purchased from Sigma, St. Louis, MO, U.S.A.; acetazolamide was obtained from Lederle Laboratories, Pearl River, NY, U.S.A. All other reagents were reagent grade.

Specimens of human liver, burn wound granulation tissue, bone, kidney and intestine were taken at autopsy of patients who had succumbed to severe thermal injury. A placenta was obtained from a normal live birth. Adult, male Sprague-Dawley rats were killed by heart puncture and exsanguination and the kidneys removed. The tissues were homogenized and the homogenates butanol extracted and dialyzed as described previously [1].

AP was assayed by a modification of the method of Bessey et al. [11], using 0.5 ml of 2-methyl-2-amino-1-propanol (AMP) buffer (Sigma 325-3), 0.75 mol/l, pH 9.4 at 37°C, containing 1 mmol/l $MgCl_2$. The final reaction volume of 2 ml included 0.5 ml of 15.2 mmol/l *p*-nitrophenyl phosphate (pNPP, Sigma 104). Final concentrations of pNPP in the kinetic experiments were 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 mmol/l. The reaction was stopped after incubation for 30 min by addition of 3 ml of 1 mol/l NaOH and the absorbance read at 410 nm using a Gilford 240 spectrophotometer with 1-cm light path. Sample absorbance divided by 0.1176 yielded activity in mI.U. In the kinetic experiments, approximately 0.5 mg of total protein with a total activity of about ten mI.U. was employed in each assay.

Inhibition studies were carried out by addition of aqueous solutions of the inhibitor prior to the addition of substrate. Inhibitor concentrations were calculated with respect to final assay mixture.

## Results

The plot of $1/V$ vs. $1/S$ yielded straight lines at several concentrations of acetazolamide ranging from 1 to 20 mmol/l (Fig. 1). The mechanism of inhibition is of the mixed (competitive and non-competitive components) type, similar to that ascribed to mafenide inhibition of AP [1], and the Lineweaver-Burk plot resembles those of furosemide (Fig. 2), ethacrynic acid (Fig. 3) and chlorothiazide (Fig. 4). There appears to be no difference in mechanism of inhibition between bone and kidney, or between rat and human. From these plots, we cal-

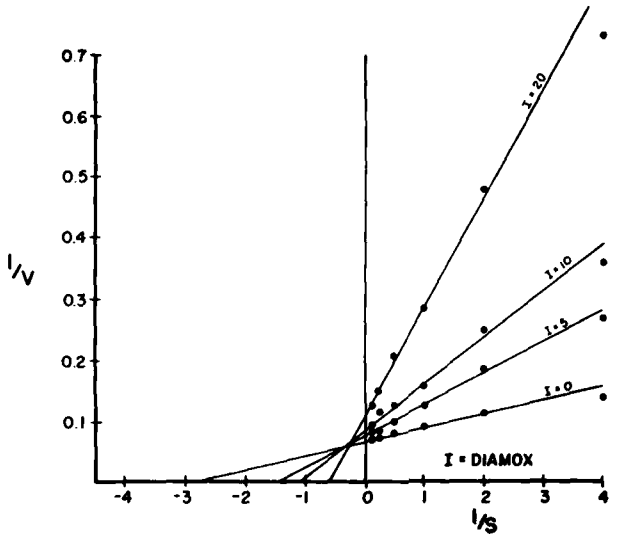




Fig. 1. Lineweaver-Burk plot (min · μmol$^{-1}$ vs. 1 · mmol$^{-1}$) of acetazolamide inhibition of human bone AP. Inhibitor concentrations in mmol/l.

culated inhibition constant ($K_i$) values of 8.4, 7.0, 2.8 and 0.1 mmol/l for acetazolamide, furosemide, ethacrynic acid and chlorothiazide, respectively.

The placental isoenzyme was relatively resistant to acetazolamide, while

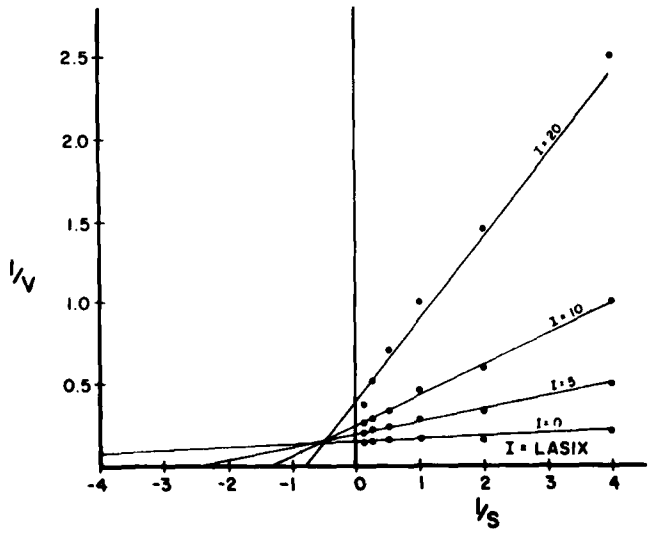


Fig. 2. Lineweaver-Burk plot (min · μmol$^{-1}$ vs. 1 · mmol$^{-1}$) of furosemide inhibition of human kidney AP. Inhibitor concentrations in mmol/l.

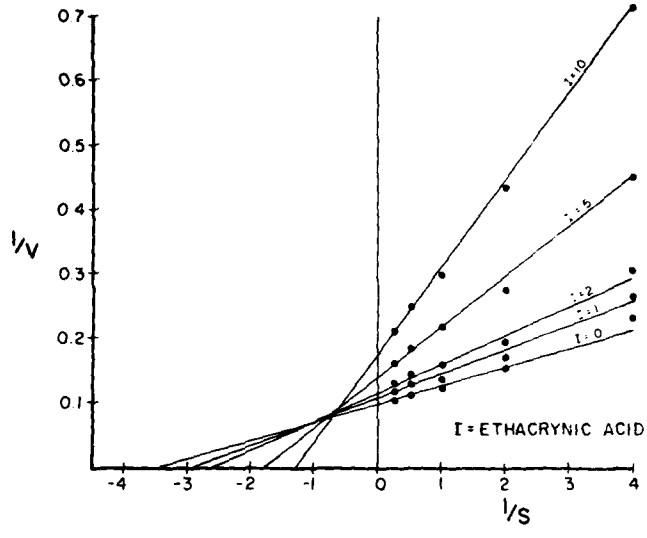


Fig. 3. Lineweaver-Burk plot of ethacrynic acid inhibition of human kidney AP. Inhibitor concentrations in mmol/l.

those of bone, intestine, liver, kidney and granulation tissue were all inhibited about 63% at 20 mmol/l acetazolamide (Table I).

There is no species difference in response of human and rat kidney AP to inhibition by chlorothiazide, ethacrynic acid or furosemide at several concentrations (Table II). Ethacrynic acid and L-cysteine separately inhibited AP

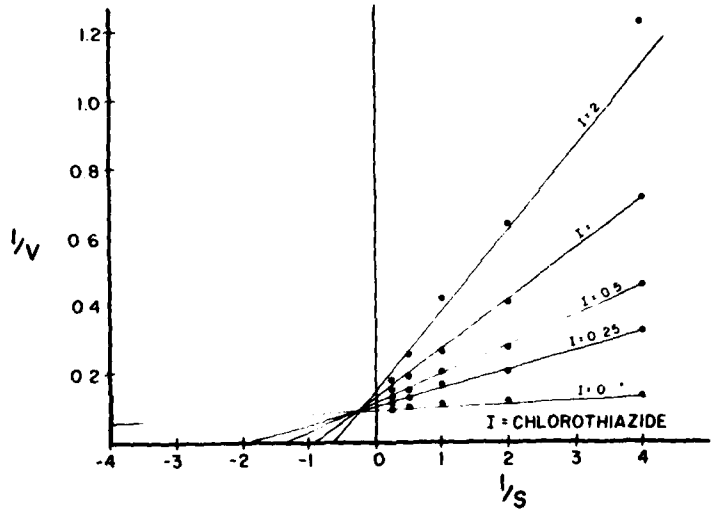


Fig. 4. Lineweaver-Burk plot (min · $\mu$mol$^{-1}$ vs. 1 · mmol$^{-1}$) of chlorothiazide inhibition of rat kidney AP. Inhibitor concentrations in mmol/l.

TABLE I

ACETAZOLAMIDE INHIBITION (%) OF ALKALINE PHOSPHATASE FROM HUMAN TISSUES

| Tissue | Acetazolamide final concentration mmol/l | | | |
|---|---|---|---|---|
| | 1.0 | 5.0 | 10.0 | 20.0 |
| Liver | 8.0 ± 4.0 | 31.7 ± 2.0 | 44.7 ± 0.6 | 63.4 ± 2.3 |
| Bone | 5.9 ± 2.8 | 30.3 ± 2.6 | 44.4 ± 3.2 | 64.1 ± 1.7 |
| Granulation tissue | 7.5 ± 3.7 | 29.6 ± 3.8 | 46.9 ± 0.7 | 63.3 ± 1.0 |
| Kidney | 0.6 ± 0.8 | 28.3 ± 2.3 | 44.8 ± 1.5 | 61.6 ± 0.0 |
| Intestine | 2.4 ± 2.2 | 30.5 ± 2.8 | 45.2 ± 3.3 | 63.3 ± 1.0 |
| Placenta | 7.5 ± 2.1 | 9.0 ± 1.9 | 13.4 ± 1.9 | 21.1 ± 2.0 |

Data represent mean ± S.D., $n = 3$.

TABLE II

COMPARISON OF INHIBITION (%) OF HUMAN AND RAT KIDNEY AP BY THREE DIURETICS

| Species | Chlorothiazide, final concentration mmol/l | | | |
|---|---|---|---|---|
| | 0.25 | 0.5 | 1.0 | 2.0 |
| Human | 16.6 ± 1.2 | 25.4 ± 1.4 | 39.1 ± 0.6 | 55.4 ± 1.6 |
| Rat | 19.8 ± 1.2 | 28.1 ± 1.4 | 45.2 ± 1.1 | 62.7 ± 1.1 |
| | Ethacrynic acid, final concentration mmol/l | | | |
| | 5.0 | 10.0 | 15.0 | |
| Human | 21.6 ± 0.6 | 52.2 ± 0.5 | 62.4 ± 0.7 | |
| Rat | 35.1 ± 0.9 | 54.4 ± 1.0 | 64.6 ± 0.3 | |
| | Furosemide, final concentration 3.0 mmol/l | | | |
| Human | 20.2 ± 3.5 | | | |
| Rat | 22.5 ± 2.1 | | | |

Data represent mean ± S.D., $n = 3$.

TABLE III

INHIBITION (%) OF HUMAN KIDNEY AP BY ETHACRYNIC ACID, CYSTEINE OR BOTH

| Inhibitor | Concentration of inhibitor, mmol/l | | |
|---|---|---|---|
| | 0.01 | 0.2 | 1.0 |
| Ethacrynic acid (EA) | 0.6 ± 0.7 | 4.0 ± 2.6 | 11.1 ± 3.0 |
| L-cysteine (Cys) | 4.3 ± 1.5 | 65.8 ± 3.3 | 92.9 ± 0.2 |
| EA + Cys | 3.5 ± 0.8 | 36.1 ± 1.1 | 74.3 ± 1.7 |

Data represent mean ± S.D., $n = 3$.

TABLE IV

INHIBITION (%) OF HUMAN KIDNEY ALKALINE PHOSPHATASE BY THREE DIURETICS

| Drug | Final concentration, mmol/l | | | |
|---|---|---|---|---|
| | 1 | 2 | 5 | 10 |
| Ethacrynic acid | 4.3 ± 1.6 | 15.6 ± 0.7 | 34.2 ± 0.1 | 49.3 ± 0.8 |
| | 1.9 | 3.8 | 7.5 | 15 |
| Furosemide | 10.5 ± 0.5 | 17.7 ± 0.5 | 36.4 ± 1.2 | 57.3 ± 1.5 |
| | 0.25 | 0.5 | 1.0 | 2.0 |
| Chlorothiazide | 16.6 ± 1.2 | 25.4 ± 1.4 | 39.1 ± 0.6 | 55.4 ± 1.6 |

$n$ = 3, data represent mean ± S.D.
Final *p*NPP concentration = 4 mmol/l.

activity, while the combination at equimolar concentrations was less inhibitory than L-cysteine alone (Table III). The inhibition of human kidney AP by ethacrynic acid, furosemide and chlorothiazide is shown in Table IV.

## Discussion

Interpretation of the significance of inhibition of an enzyme whose physiologic role is poorly understood, is obviously difficult. Although the compounds tested are diuretics of diverse chemical structure, several arguments can be made against a conclusion that alkaline phosphatase plays a significant role in diuresis.

The kidney isoenzyme is no more sensitive to the action of these diuretics than are those from liver or bone. It has been reported that furosemide and ethacrynic acid were equally effective diuretics in man [10], but that rats were relatively insensitive to ethacrynic acid [11]. If the inhibition of AP played a major role in diuresis, one might expect a difference in the response of rat and human AP to ethacrynic acid. But our data show no species difference in inhibitory responses. The doses of diuretics which significantly inhibit AP in vitro are markedly higher than those which are effective in vivo. It should be remembered, however, that the test conditions are non-physiologic, in terms of substrate, pH and ionic strength, and that the enzyme has been solubilized from the cell membrane. The same caveat applies to comparisons of $K_i$ or $K_m$ values, derived from these experiments, to those reported in other systems.

The role of AP in normal physiologic processes, development or healing has not been defined. Perhaps studies of its response to inhibitors on stimulators may help to elucidate its function. It may be useful, after observing inhibition by non-physiologic drugs such as the diuretics employed in this study, to investigate the effects of physiologic analogues of known inhibitors.

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